REPORT OF THE CROSS-SPECIES INFECTIVITY AND PATHOGENESIS MEETING

JULY 21 & 22, 1997

Dr. Harold E. Varmus, Director of the National Institutes of Health (NIH), opened the 2-day Cross-Species Infectivity and Pathogenesis Meeting sponsored by NIH, the Food and Drug Administration, the Centers for Disease Control and Prevention, and the Health Resources and Services Administration on July 21 and 22, 1997. Dr. Varmus asked the 230 attendees from several countries to consider the state of medical transplantation today. For approximately 50,000 patients currently waiting for transplant organs in the United States, there are only about 5,500 cadaver donors annually. Estimates of optimal annual recruitment of cadaver donors are still only around 10,000. This organ shortage provides incentive for the option of xenotransplantation, using nonhuman species as organ donors for human beings. Moreover, the advent of methods to genetically alter donor animals to make them more compatible for host acceptance and the use of cyclosporin to inhibit organ rejection have boosted hopes that humans could successfully receive organs from donor animals such as baboons and pigs.

Although the Public Health Service has published draft guidelines for xenotransplantation in the *Federal Register*, Dr. Varmus invited meeting participants to consider several issues to help decision-makers fine-tune the proposed guidelines. First, he requested that scientists summarize relevant zoonoses, infections shared by both humans and other vertebrates, which may be contributory in xenotransplantation. Second, he asked them to consider the effect of recipient immuno-suppression or donor genetic manipulation in the context of zoonoses. Third, scientists were asked to think about any potential threat posed by donor endogenous infectious agents that have not yet been linked to clinical disease but may cause disease during xenotransplantation. Fourth, how can known agents be monitored and unknown agents be detected? And fifth, what is the risk to the public exposed to xenotransplant recipients? These issues will shape the assessment of risk/benefit ratios that determine how and if xenotransplantation proceeds.

Endogenous Viruses Properties and Cross-Species Transmission

Dr. John M. Coffin presented the plenary address on the history and study of endogenous retroviruses, including those that have previously entered the human population from animal reservoirs. Endogenous proviruses are fossil traces of ancestral exogenous retroviral infections. They can comprise up to about 0.5 percent of the DNA for a given species. Major differences exist between endogenous proviruses that entered the germ line prior to and after speciation. While older proviral sequences exist in all species, more recently acquired endogenous proviruses exhibit an "erratic species distribution." Ancient endogenous proviruses evolved so as to mirror phylogenic evolution, such that sequence analysis of a specific ancestral provirus would have the same evolutionary splits as a standard phylogenic tree. More recently integrated endogenous proviral sequences are distributed among species so as to reflect when and what the retrovirus originally infected. A provirus could exist only in humans, chimpanzees, and gorillas and not in other apes or old world monkeys if it integrated around 6 million years ago at about the point of human, chimpanzee, and gorilla divergence, whereas provirus inserted 30 million years ago, prior to divergence, would be distributed among all of these primates.

More recently acquired endogenous proviruses are polymorphically distributed, even within a species. Therefore, a donor subspecies could be selected or selectively bred to eliminate proviral sequences believed to pose an infection risk in xenotransplantation. Some endogenous proviruses confer resistance to infection with similar exogenous retroviruses and thus offer an advantage to species or individuals with them. Dr. Coffin used the example of C type provirus in mice to show how polymorphisms could be used as markers during selective breeding to remove unwanted endogenous provirus.

It would be important to select out polymorphic endogenous proviruses that are or could be associated with diseases. Pathogenic murine leukemia virus (MLV) is derived from a relatively benign endogenous MLV that underwent recombination with a xenotropic provirus that is biologically active but noninfectious in mice. The new virus has novel long-terminal repeats (LTR) sequences that make it pathogenic in the thymus. A polymorphism in the viral receptor prevents infection by xenotropic virus in most inbred strains of mice. However, xenotropic MLV can infect cells from some mouse subspecies and most other mammalian species, including humans. Human tumor cells grown in nude mice (which carry the BXV-1 locus that gives rise to xenotropic virus) became infected and have given rise to reports of "human" tumor viruses.

In the context of xenotransplantation, Dr. Coffin outlined the following issues to be assessed. Not all-possible donor species carry infectious endogenous provirus. Of those that do, it must be determined whether the infectious provirus can infect human cells. If it can, then it is "probably more or less inevitable" that some human cells will become infected through xenotransplantation. Recent endogenous proviruses are readily activated by DNA demethylation and may lead to viremia. The big question is "What happens after infection?" If a recipient's immune system can wipe out the infection quickly or the infection causes a pathogenesis that leads to a tumor 20 years later, then this may be an acceptable risk for someone who needs an organ to survive. The courses of any potential infection and subsequent pathogenesis in an immunosuppressed transplant recipient need to be determined. And it is critical to understand the infection risk to those coming

in contact with the recipient. Furthermore, it must be clarified how a potentially unknown infectious agent would be detected.

PANEL SESSION I: CROSS-SPECIES TRANSMISSION SPECIES SPECIFICITY AND TROPISM

Factors Which Influence Pathogen Entry into Hosts (e.g., Viral Receptors)

Dr. Preston Marx spoke on ***SIV Infection of Macaques: A Model of Cross-Species Transmission and Pathogenesis***. The infection of Asian rhesus macaques by simian immunodeficiency viruses (SIVs) derived from sooty mangabeys is a model for cross-species viral infection. SIV sooty mangabey (SIV/SM) has been conclusively shown to be the origin of SIV/MAC. While no illness due to SIV/SM infection has been detected in sooty mangabeys, its transmission into the cross-species host (macaque) induces AIDS fairly rapidly. SIV/MAC uses "analogous cell surface receptors in either host." SIV/PBJ is another pathogenic SIV derived from sooty mangabey. PBJ contains a mutation in the *nef* gene that allows the virus to induce an acutely lethal gastrointestinal (GI) disease. Animals that survive the initial GI attack later develop AIDS.

SIV/MAC has been developed as a model of mucosal transmission in order to understand early transmission pathogenesis. An aim is to use this model to develop possible viral vaccines. Characteristics of SIV/MAC include that it is secreted in body fluids and crosses genital and rectal epithelium. In addition, cell-free transmission of SIV is much easier than cell-associated SIV transmission. Moreover, the intact vaginal mucosa is a partial, not complete, barrier to transmission and some naturally occurring attenuated SIVs transmit without causing disease.

Vaginally introduced virulent SIV/MAC strain 239 exhibits a clear distinction between rapid and slow progressors in the macaque. Rapid progressors have plasma RNA levels that achieve 10^6 to 10^7 copies, have no detectable antibodies, and die in 1 to 6 months, whereas slow progressors generate a strong antibody response and generally live 2 to 3 years, with some animals surviving for 6 to 7 years.

Target regions of the female reproductive tract include the vaginal mucosa, vaginal epithelium, the ectocervix, and the transition region where the tissue goes from stratified squamous epithelium to cervical single-cell epithelium. Dendritic cells, antigen-presenting cells, of the cervix have been shown to be an SIV/MAC target both *in vitro* in humans and *in vivo* in macaques. Dendritic cells drain to lymph nodes, providing direct shuttles for virus to enter and replicate in the immune system. SIV-positive dendritic cells are seen in the vagina at day 2 post-infection by *in situ* PCR.

"Receptors and coreceptors are interchangeable *in vitro* between macaque and human cells and between SIV and HIV." CCR5 and CXCR4 are coreceptors generally used by HIV-1 strains that are macrophage (M) tropic or T-cell (T) tropic. However, CCR5 is the coreceptor for SIV strains that are either M tropic or T tropic: SIV does not use CRCX4. CCR5 is highly conserved across primate species and will act as a coreceptor for SIV, HIV-1, and HIV-2 according to tropism. Yet, human cells with a deficient CCR5 support SIV. Hence, there is clearly an unknown coreceptor for SIV.

Dr. Marx has also found a new red capped mangabey virus, an SIV with the HIV-1 pol gene. It grows in human and rhesus peripheral blood mononuclear cells (PBMCs) and it does not use CCR5! This is the first known monkey virus in the HIV-1 lineage.

Propagation and Replication Across Species Barriers

Drs. Robin Weiss, Carolyn Wilson, David Onions, and Jonathan Stoye each spoke on this topic.

Dr. Weiss spoke on endogenous ***Retroviruses That Jump Host Species***. His early research revealed that an endogenous viral envelope gene could complement an envelope-deficient sarcoma virus and thus augment infection. The subsequent discovery of reverse transcriptase clarified how this could come about.

Endogenous retroviruses generally do not cause disease in their host population, because "the host, which has acquired the virus genetically, evolves resistance to it." Murine leukemia virus demonstrates this. However, host species can change over time. One feline endogenous virus entered the cat population after exogenous infection from a baboon endogenous C type retrovirus. Moreover, the baboon endogenous retrovirus appears to have resulted from a recombination between an initial C type virus and a portion of a D type simian retrovirus. Furthermore, this virus was first identified incorrectly as a human retrovirus (*Nature*, 1971) when human tumor cells were grown as a xenograft in fetal cat brains. The virus, which is present in all cats, came out in human cells, since the "human cells did not restrict the replication of this virus."

More current work growing human tumor cells in nude and other immunodeficient mice demonstrates host into graft retrovirus colonization. Approximately one in three serially transplanted tumors are productively infected with these xenotropic viruses. The question to address in the context of xenotransplantation is whether graft into host virus transfer is likely.

Researchers are beginning to assess pig viruses for pig into human viral transmission. A porcine kidney cell line PK15 releases C type viral particles that can infect pig testes, ST-IOWA cells, mink cells, and human 293 cells. However, most other human cells are not infected. Yet, cocultivation with irradiated PK15 cells led to infection of a greater number of human cell types. On the other hand, virus from swine kidney cells (MPK) infects pig cells but not human cells. Pigs appear to have several copies of this endogenous C type virus, but there is some polymorphism that might make it possible to breed out this virus.

It is possible that genetically modified pigs would be more capable of cross-species viral transmission. Abrogation of hyperacute rejection will make pig enveloped viruses more resistant to inactivation by human complement, and some viruses may be preadapted for transfer to humans. In fact, retroviral vectors produced in mouse or dog packaging cell lines are complement-inactivated when introduced *in vivo*. This inactivation occurs by the exact same mechanism that causes hyperacute rejection in xenotransplantation. Humans are genetic knockouts for the α1,3-galactosyl-transferase gene and make antibodies against this enzyme. It is under investigation whether viruses released from cells of transgenic pigs with human cell membrane proteins are more resistant to inactivation by human complement. This could affect the selection of pigs as xenotransplant donors.

Dr. Wilson spoke on ***Induction and Isolation of a Retrovirus with a Human Host***. NIH mini-pig peripheral blood mononuclear cells (PBMC) were used to develop an *in vitro* model that would mirror a xenotransplant setting. PBMCs mitogenically activated with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) had a reverse transcriptase (RT) activity peak at 5 days post-activation. These PBMCs were cocultivated with human 239 cells and ST-IOWA cells after the 5-day period. Both cell types showed increasing RT activity after a lag period between 20 and 40 days as well as a productive infection that spread efficiently. Thus, it has been shown that infectious retrovirus can be isolated from at least two separate strains of the NIH mini-pig by mere mitogenic stimulation of PBMCs. Virus released from these activated cells directly infected both pig and human embryonic kidney cells.

The RTs isolated from three pig-infected cell lines showed a high degree of homology. ST cells infected with NIH mini-pig virus, ST cells infected with Yucatan PBMCs, and 293 cells infected with NIH mini-pig virus have identical RTs at the amino acid level and there is only one nucleotide difference in ST Yucatan RT compared to the other RTs.

Dr. Onions spoke on ***Cross-Species Transmission of Viruses: Implications for Xenotransplantation with Porcine Tissue***. Zoonotic viruses from pigs that can replicate in human cells are of particular concern in the context of xenotransplantation. In a xenograft, viruses (such as herpes and paramyxoviruses) may also spread by cell-to-cell contact and syncytia formation. The H1N1 swine flu virus caused one of the greatest pandemics of this century in 1918 and 1919. With regard to influenza, in fact, pigs appear to act "as a mixing vessel for both avian and human viruses; avian viruses do not seem to go directly to man." Viral groups associated with cross-species transmission include parvoviruses, coronaviruses, rotaviruses, influenza viruses, retroviruses, adenoviruses, morbillivirus, herpesviruses, and papillomaviruses.

Reassortment and emergence of novel porcine viruses continues today. Porcine reproductive respiratory syndrome virus, which probably arose from a rodent arteritis virus in the past decade, causes a new emerging disease in pigs. Since influenza virus H1N1, which is a constantly changing virus, is still endemic in the pig population, it is possible that these two viruses could further combine.

The alpha herpesvirus, otherwise called pseudorabies, seems to have limited cross-species transmission. The GE glycoprotein of this virus is important for it to exit the cell. While this virus replicates in human cells, "there is no evidence that it is zoonotic."

It has been observed that many large complex viruses have evolved genetic mechanisms for modulating immune responses that would otherwise attack them. Some of them carry genes that block tumor necrosis factor induction of infected cell apoptosis. And the gamma herpesviruses carry an IL-10 homologue, which switches the anti-viral, cytotoxic T helper 1 response to an antibody producing T helper 2 response.

Canine adenovirus 1 exhibits another type of transmission block. While the degree of homology between some regions of this virus and human adenovirus 5 is high enough to indicate that the canine virus could infect humans, the two viruses' E3 regions contain immune-modulating genes that have hardly any identity. Hence, even if the canine virus infected humans, it probably could not establish a persistent infection. In sharp contrast, a significant cross-species jump occurred

with adenovirus 76. Adenovirus 76 is normally found in ducks, but contaminated a manmade chicken vaccine and killed hundreds of millions of chickens. This virus is now established as a chicken-to-chicken infection.

One gamma herpesvirus, bovine herpesvirus II, is a lymphoma-inducing fatal virus for cows. It originated in sheep where it appears to do no harm, but it jumped host species to cows when sheep and cows were grazed together in the same pasture. This would be a dead-end transmission.

Some porcine viruses could be unproductive but also be oncogenic. Viruses that are important in veterinary medicine should be tested for in donor animals. But, “What do you do about the unknown?” The pig could carry a gamma herpesvirus that is unknown due to its benign state in pigs, but it could "come out" in xenotransplantation.

The condition of decreased immune surveillance could lead to graft damage. Porcine cytomegalovirus may act this way, and human viruses like flu, adenoviruses, rhinoviruses, or hepatitis C could infect pig cells.

Minor changes in some viruses can result in a change in tissue or species tropism. Coronaviruses are particularly susceptible to these events. In addition, one feline parvovirus changes into a canine parvovirus with relatively few nucleotide changes. FeLV-A, feline leukemia virus A, does not infect human cells, but it can undergo a few mutations and deletions to become FeLV-C and gain the ability to infect human cells. And in a pig parvovirus, a five amino acid mutation changes a nonpathogenic virus into one that is highly pathogenic.

Hysterectomy or hysterotomy followed by early or segregated weaning for consecutive generations is proposed as one method to create optimal xenotransplant donor pigs. This would require close monitoring to prevent the reintroduction of unwanted viral strains through various vectors, including the staff and the housing. Viruses that cross the placenta require special attention. But endogenous retroviruses stand out as the current major barrier.

“The dooms day scenario is . . . that you take a porcine organ, you transplant it into a person, a porcine cell expresses a retrovirus, it infects a human cell . . . (and) produces a virus capable of transmission to the general public. Complex recombinations do occur, but they are not common.”

Porcine kidney PK15 cells do express a retrovirus capable of infecting human cells. The viral polymerase (*pol*) region has the highest identity, and the envelope (*env*) region has significant identity to human retroviral equivalents. The porcine endogenous virus envelope does confer the ability to infect human cells, but it is not very efficient. Scientists have developed an ELISA assay to detect antibodies to this virus and have the RT-PCR tools to detect viral expression; therefore, it is likely that this virus would be detected prior to use of porcine organs.

In monitoring possible infection of xenotransplant recipients, the infectious state needs to be considered. For example, FeLV-A can infect a cat in such a way that the cat has both antigen and antibody, but the virus may be sequestered and not appear in PBMCs. Moreover, a cat may appear to be in recovery but have provirus-positive cells within the bone marrow. Therefore, it will be important to have a range of tests to determine infection states.

To determine whether xenotransplantation is safe, it needs to be established whether porcine infectious virions are produced *in vivo* in pig organs and whether they infect primates, and it will be necessary to look for antibody as well as PCR positivity. The safest solution is to breed pigs lacking expressible provirus or use gene knockouts to get rid of unwanted sequences. Potentially, xenograft recipients could also be vaccinated against unwanted viruses.

Dr. Stoye spoke on ***Distribution and Host Range Properties of Two Classes of Pig Endogenous Retroviruses***. Endogenous retroviruses became established in the germ line after germ cell infection; thereafter, they are inherited as Mendelian genes. Host species evolved mechanisms, such as Fv1 and Fv4 genes, to control replication of these endogenous sequences; so it is probably important to restrict their replication. While most of these viruses are defective, some of them can give rise to infectious virus or "contain partial gene products that can recombine with infecting exogenous retroviruses." Endogenous retroviruses produced by the pig kidney cell line, PK15, were examined. Studies were focused on the viral envelope gene, because this is the region that determines the retroviral host range.

Two PK15 porcine endogenous retrovirus (PERV) strains, PERV-A and PERV-B, were isolated. They belong to the mammalian C type retrovirus family. PERV-A and PERV-B showed 92 percent amino-acid identity in the transmembrane region of the envelope protein. Analysis of the cDNA clones isolated from pig cells showed that 29 out of 32 clones were PERV-A, and only 3 clones were PERV-B. However, cDNA clones from human 293 cells were exclusively PERV-B. So, initially, PERV-A was thought to be ecotropic, and PERV-B was believed to be xenotropic. Though PERV-A and PERV-B are virtually identical in the transmembrane (TM) region, they have significant differences in the SU region (cell attachment region) of the *env* protein. The proviruses have major differences in the VRA, VRB, and polypurine-rich regions, suggesting that they will bind two different receptors.

Neither PERV-A nor PERV-B was present in uninfected human 293 cells. RT-PCR studies revealed that 293 cells infected with PK15 expressed both viruses, which showed that PERV-A was transferred with PERV-B in these cells. PERV-A could be a pseudotype of PERV-B. Alternatively, both viruses can infect human cells. Recent experiments to generate cell lines infected with only one of these proviruses are consistent with the latter possibility. It has now been shown that both PERV-A and PERV-B can infect pig and human cells, i.e., are polytropic.

It would be desirable to selectively breed pigs lacking viruses capable of infecting human cells. This will be difficult, because all pig varieties tested contain 20 to 40 PERVs, and at least eight PERV proviruses are shared among all varieties of pigs tested. The Meishan pig does seem to have slightly fewer, so it may be a good breeding candidate. Yet, it is not clear what portion of these endogenous proviruses can give rise to infectious virus. This needs to be determined.

The overall picture indicates that retroviruses present in donor pig herds will be expressed in transplant tissue. It also seems likely that the recipient will become infected, particularly with immunosuppression to prevent organ rejection; the immunosuppression will allow the virus to evade human immune responses. Currently, the key issues appear to be whether infection leads to high levels of viral replication in the transplant recipient and, if so, whether any pathology results. Infection will most probably occur without associated pathology. However, infections could result in pathology that manifests many years after the transplant; some may give rise to cancers. The

greatest risk would be the generation of “a highly transmissible pathogenic virus, which could affect all of us.” While this may be possible, it seems very, very unlikely.

Panel Discussion I:

An open panel discussion with comments and questions from attendees ensued. (1) The concern about generating knockout pigs was allayed. (2) The relationship between viremia and virus transmissibility was addressed. Dr. Onions took the position that viremia is a “danger point” from the standpoint that a “reasonably hot” virus that is constantly bombarding cells will probably eventually hit and activate an oncogene, not because viremia will “itself lead to transmissibility.” (3) It was also asked why the infectivity of PK15 virus has not been explored in human PBLs. This work, as well as determining the virus’ specific cellular tropism, is planned.

Factors Which Restrict Post-Entry Stages of Pathogenesis

Drs. Michael Malim and Beatrice Hahn gave presentations on this topic.

Dr. Malim spoke on ***Analysis of Lentivirus VIF Protein Function***. Virus infectivity factor (VIF) is a gene encoding one of the six major accessory proteins of HIV-1. This protein is "probably a critical determinant in the ability of primary lentiviruses to switch host species." If Vif protein is absent during HIV-1 late stages of virion assembly and/or maturation, then the virus becomes essentially noninfectious. While Vif is usually required for viral replication, certain T cell lines appear capable of replicating Vif-deleted virus.

It is proposed that Vif is necessary to maintain post-penetration assembly and prevent degradation of viral nucleic acids. A number of analyses have confirmed that Vif is present in large quantities at the plasma membrane where virus is assembled. An average 30 to 80 molecules of Vif are packaged into each HIV-1 virion. Gag and Vif concentrations are roughly equivalent, and they are co-localized in productively infected cells. "Vif may play a structural role rather than a catalytic role in virus assembly."

Normal Vif-competent virions develop a pre-integration complex that leads to reverse transcription and infection. Vif-deficient infection is believed to lead to an aberrant pre-integration complex, which cannot complete reverse transcription.

When Vif-deleted HIV-1 provirus was co-transfected with Vif alleles from various primate immunodeficiency viruses, only some alleles effectively complemented infectivity. In particular, Vif alleles of SIVagm at SIVsyk did not function. The various alleles were only 20 to 30 percent identical. Experiments undertaken to clarify why certain alleles complemented and others did not revealed that Vif function was somehow host cell species restricted. Furthermore, studies with murine leukemia virus showed that HIV-1 Vif protein can affect heterologous viral replication. This indicates that Vif may alter the cellular environment allowing for infectious retroviral particle production. Hence, individual viruses would only be effectively transmitted and generate an infection in human cells if its Vif was competent in those cells. This has broad implications for determining viral transmissibility in xenotransplantation.

Dr. Hahn spoke on ***Cross-Species Transmission of Primate Lentiviruses***. Phylogenetic analyses of HIV-1 and HIV-2 provide strong evidence that both of these virus groups are the result of zoonotic transmissions of lentiviruses from naturally infected primates to humans.

The sooty mangabey virus is the source of HIV-2. Because sooty mangabeys are hunted and kept as pets in some regions of West Africa, there is ample opportunity for contact with humans. Possibly six independent transmissions of SIVsm strains into humans have occurred. Some of these have become epidemically spreading pathogens, while others have only been identified in single individuals. It will be important to determine why the latter viruses appear to be unable to spread. They could be genetically so divergent that they fail to grow in humans, or they could require repeated passage in the new host for rapid adaptation.

There is genetic evidence that SIVcpz-infected chimpanzees were the source of HIV-1 in humans. However, the number of naturally infected animals appears to be too low to represent a sensible reservoir. Possibly, a third, as yet unidentified, primate species has transmitted virus to both humans and chimpanzees.

In the wild, African green monkeys are the most commonly SIV-infected primates, but there is no evidence that cross-species transmission into humans has occurred. This may be because the SIVagm proteins do not function properly in human cells, as detailed in Dr. Malim's presentation. For example, Vpr causes an arrest of the cell cycle of infected cells, and this function is conserved among all primate lentiviruses. However, the G2 arrest function is species specific, i.e., the SIVagm Vpr does not work in human cells. The ability of viral gene products to interact with human proteins could thus represent "predictors of transmissibility."

Finally, all naturally occurring SIVs fail to cause immunodeficiency in their natural host. It will be important to determine why cross-species transmission turns these viruses into potent pathogens.

Mutation Rate, Recombination, Multi-gene Reassortment, and Pseudotyping

Drs. Kathryn V. Holmes and Ralph Baric spoke on this topic.

Dr. Holmes and **Dr. Baric** addressed the species specificity of coronavirus receptors. Most of the talks in this meeting address concerns about DNA viruses or retroviruses that may be incorporated in the genome of donor cells in a xenotransplant and may be activated upon transplantation to cause disease in the transplant recipient. Cross-species infection with many groups of RNA viruses other than retroviruses is also theoretically possible, even though these viral genomes do not become incorporated into the host DNA. RNA viruses have very high mutation rates and many can cause persistent infection *in vivo*, leading to the accumulation of mutant viruses, some of which might be transmissible to a new host species. While such a species-jumping event might occur very rarely, a host range mutant virus present in a xenotransplant donor could potentially initiate infection in an immunosuppressed recipient, or, in the worst case scenario, outbreaks of infectious disease in the recipient species. It is therefore important to understand how an RNA virus can mutate to achieve an extended host range.

Dr. Holmes spoke on ***Cross-Species Infection with Coronaviruses***; a class of large (32kb) enveloped RNA viruses. Several coronaviruses cause persistent infections. Most individual coronaviruses infect a single species and cause enteric, respiratory, systemic, or neurological diseases, in acute or persistent infections.

Coronaviruses have a high mutation rate and a very high recombination rate. They mutate at a rate of about 1 in 10,000 nucleotides, which translates to an average of about three mutations per genome. Furthermore, they can recombine with different strains and, rarely, acquire features from other viruses, such as HE from influenza C virus. Thus, the replication of coronaviruses gives rise to multiple viral quasi-species, with different biological properties.

Dr. Holmes' research focuses on mouse hepatitis virus (MHV), a group 2 coronavirus, which attaches to host cells using the spike glycoprotein (S) to bind to specific cellular receptors. Most group 2 coronaviruses express a hemagglutinin-esterase (HE) glycoprotein, which is derived from influenza C HE. HE binds to 9-0-acetylated sialic acid moieties on the cell membrane. The viral S protein binds to specific murine glycoprotein receptors with differing efficiencies. MHV receptor (MHVR), a biliary glycoprotein in the immunoglobulin superfamily, is the first identified natural receptor for MHV. Other related murine glycoproteins with weaker receptor activity include BGP2 and brain carcinoembryonic antigen (CEA), an anchorless receptor. Soluble recombinant MHVR-related glycoproteins can neutralize MHV strains.

Aminopeptidase N (APN) is the receptor glycoprotein used by several group 1 coronaviruses. Expression of human APN (hAPN) confers susceptibility to human coronavirus HCV-229E on resistant cells such as hamster cells, but hAPN is not a receptor for the related porcine virus TGEV. Thus, the species specificity of infection is determined by the virus-receptor interactions. However, expression of recombinant feline APN makes hamster cells susceptible to infection by porcine, human, and feline coronaviruses. So the feline APN (fAPN) is a more universal receptor than hAPN. The domains of the receptor and the virus S glycoproteins that interact are being

determined to find out what features of the receptor glycoprotein determine the species specificity of coronavirus infection.

Many coronaviruses can cause persistent infection *in vivo*. Long periods of viral infection, coupled with the high mutation and recombination rates of these viruses, increase the likelihood that a virus mutant that has an extended host range might arise. For example, MHV strain A59 does not infect hamster cells; however, virus from the 600th passage of mouse cells persistently infected with MHV-A59 (MHVpi600) can infect hamster cells, as well as bovine, feline, human, and rat cells. These cells do not express MHVR or other known MHV receptors, so the receptor specificity of the viral S glycoprotein must be altered. An antireceptor antibody that blocks MHV-A59 infection of murine cells cannot completely block infection with MHVpi600. So the new virus strain is either using the old receptor differently or using a different receptor on murine cells.

Some RNA viruses may cause persistent inapparent infection of donor tissues. Therefore, in the context of xenotransplantation, donor sera should be tested for antibodies to viruses common to that species that might cause persistent inapparent infection and a small amount of the transplanted tissue should be frozen for later study. The recipient should be isolated after the transplant until it can be seen if he or she becomes ill or seroconverts to a virus that derived from the donor tissue. If the recipient contracts an unexplained illness, the presence of a virus in the donor tissue can be explored. Thus, sensitive tests are needed for detection of donor viruses in transplanted tissues and for seroconversion of recipients to viruses from the donor species.

Dr. Baric spoke on ***Molecular and Evolutionary Mechanisms of Virus Cross-Species Transmission***. Coronavirus replication is characterized by high mutation (10^{-4}) and RNA recombination frequencies (about 20 percent), suggesting that these viruses are well positioned to adapt rapidly to a changing ecological niche. Because species specificity in this family of viruses is almost exclusively mediated at the level of entry, coronaviruses are good model systems to study receptor molecule lineages that regulate virus cross-species transmission. Molecular and evolutionary mechanisms of virus cross-species transmission are being studied in a model system that may reflect conditions in human xenograft recipients. This model system consists of mouse hepatitis virus co-cultivated in a mixed population of permissive (mouse) and nonpermissive (hamster) cell lines. While hamster cells completely restrict the replication of the parental wild-type viruses, a passage series from the model cell mixture yielded MHVH1 and MHVH2 variants that could efficiently replicate in hamster cells.

MHVH2 could also replicate efficiently in human cell lines, demonstrating that virus mutants could emerge with broad host range specificity from mixed cell populations. In addition, MHVH2 does not use the same biliary glycoprotein (Bgp) receptor (MHVR) as the parental strains; rather, it seems to use $Bgp1^b$ and Bgp2 glycoproteins as receptors for docking and entry into mouse cells. Thus, cross-species transmission in mixed cell populations may also result in altered virus virulence, tropism, and pathogenesis in the original host.

MHVR is a member of the highly homologous carcinoembryonic (CEA) gene family. Humans are known to have about 22 different genes in this family in their genome, including the biliary glycoprotein homologue of MHVR. Although it is unclear exactly which human CEA glycoprotein family member functions as a receptor for MHVH2 entry into human cells, antiserum against the human CEA glycoproteins blocks virus replication, suggesting that phylogenetic

homologues of the normal receptor function as natural conduits of virus cross-species transmission in mixed cell cultures.

The evolutionary mechanisms by which viruses adapt to mixed host cell populations have been a matter of intense investigation. Neutral allele theory proposes that “most mutations are deleterious, that advantageous mutations are very rare, that deleterious mutations are removed by purifying selection, and that less important portions of molecules evolve faster than more important portions of molecules.” This model supports the concept of a constant molecular clock that applies nearly equivalent mutational pressure over time to yield phylogenic variation patterns. This would result in more silent mutations than mutations in active proteins and accounts for most, but not all, of the genomic polymorphisms noted at the molecular level.

To account for other observations in viruses that cross species barriers, the episodic evolution theory proposes that mutation rates are relatively constant until environmental condition change, resulting in “dramatic shifts in the selection pressure.” This would support a mutation pattern with more mutations maintained in active proteins than in silent regions not under direct selection.

Molecular analysis of the MHVH2 variant reveals that mutations in the S and HE attachment and entry glycoproteins are fixed at significantly higher rates as compared with mutation rates measured elsewhere in the viral genome. Mutations in other regions of the virus are completely silent and did not affect tropism. Importantly, no silent mutations occurred in the S glycoprotein, and only two of eight HE mutations were silent. These observations suggest that cocultivation of viruses in mixed cell populations from different species appears to support “explosive episodic evolution and positive Darwinian natural selection.”

Based on this model, xenotransplantation will probably support the cross-species transmission of animal viruses. The viruses seem to evolve with selective pressure along phylogenic homologues of the normal receptor. This could “remodel virus receptor interactions in the original host, allowing for the emergence of new human viruses that may be maintained in animal reservoirs.”

Because relatively few mutations are needed to expand virus host range, data suggest either that cross-species transmissions are more prevalent than normally believed or that additional barriers to cross-species transmission exist. Depending on the virus family, such barriers may exist at the level of each individual host, at transmission between hosts, or at an ecological level.

Panel Discussion II:

An open panel discussion with comments and questions from attendees ensued. (1) It was considered that endogenous Vif could establish or enhance viral replication of other retroviruses already present in a graft recipient. (2) It was expressed that transgenically altering pigs to express certain receptors, such as Gaf, could make the pig tissues more susceptible to viruses that use that specific receptor. (3) It was pointed out that in order to survey for possible coronavirus infection, it will be important to directly sample tissue; testing peripheral blood would not render a complete assessment of potential infection. This emphasizes the need for good diagnostic tests.

PANEL SESSION II: CROSS-SPECIES TRANSMISSION MECHANISMS OF PATHOGEN ADAPTATION

Mutation Rate, Recombination, Multi-gene Reassortment, and Pseudotyping (cont.)

Drs. Colin Parrish and Robert Webster spoke on this topic.

Dr. Parrish spoke on ***The Acquisition of an Extended Animal Host Range by a Virus, and Its Subsequent Evolution and Host Adaptation The Example of Canine Parvovirus***.
Parvoviruses are widespread in animals and insects. Yet, they have not been found outside of the carnivore order. These DNA viruses have very stable virions: it is thought that they may be viable for months or years in a cool dark environment. The route of transmission is usually fecal-oral, most do not form persistent infections, and effective immunity to them is generally antibody mediated. However, at least one canine parvovirus can attack the intestine, erode the villi, and kill a dog due to loss of gut osmoregulation.

Canine parvovirus (CPV) was seen to cause a new disease in dogs in 1978. The original variant, CPV Type II, did not replicate in dogs, but it was replaced by another antigenic type that could replicate in dogs. Since then, it underwent a series of adaptive changes that make it more transmissible between dogs. It spreads quite effectively and entered wolf and coyote populations. Another variant arose around 1984 and has since become widespread due to a selective advantage. The variants do not have a large number of genetic variations; they just have the right type of variations to make a difference. However, each variant is derived from the same ancestral sequence.

Nucleotide sequence variation in the capsid protein gene of several variants reveals that most of the changes occurring are non-synonymous, giving rise to changes in the encoded amino acid at that site. In addition, they exhibit a higher ratio of transversions (purine to pyrimidine or pyrimidine to purine) to transitions (purine to purine or pyrimidine to pyrimidine) than normal. Usually, transitions occur about ten times more frequently than transversions. This indicates that the variant mutations are being selected and are not just mutually evolving.

The mutation rate is roughly 2×10^{-4} nucleotides per year. While this is about tenfold slower than some RNA viruses studied in epidemic situations, it is still about a thousand fold faster than most DNA viruses.

There also appears to be a codon bias, which is not yet understood. However, most of the changes that appear in the capsid protein are on either the inner or outer exposed surface. They are not randomly dispersed throughout. This also supports the claim that the changes are being selected.

Viral host range cannot necessarily be predicted in advance; it has to be defined empirically. Feline parvovirus (FPV) probably acquired its ability to infect dogs by gaining an ability to infect new tissue: it gained an extended tissue tropism to the intestines. Although FPV can grow in dog thymus cells, unless it is taken up in the intestine, infection would not occur. Once the virus

incurred intestinal tissue tropism, it could be transmitted between dogs. This extended tropism is the result of only two amino acid sequence substitutions, which are governed by two underlying nucleotide substitutions. But it is still unclear how this allows for intestinal tropism.

Dr. Webster spoke on ***Transmission of Influenza Virus Between Species***. Influenza A viruses are RNA viruses with many quasi-species. In addition to human influenza virus, equine, porcine, avian and other species-specific strains are recognized. Avian influenza viruses are perpetuated in aquatic birds and occasionally transmit to other host species. Periodically they can transmit to humans indirectly, through pigs. Three pandemics of influenza A in humans in this century developed along this route. In addition, pigs maintain descendants of the famous Spanish flu influenza, some of the earlier human viruses, and recently transmitted avian viruses.

The hemagglutinin and neuraminidase glycoproteins of influenza can accommodate an enormous number of changes. Mechanisms of influenza variation include genetic drift (there is no RNA polymerase to correct errors); genetic shift; reassortment; direct host range transmission; and true intergenic recombination.

There are 15 hemagglutinin subtypes of influenza viruses. All transmit to turkeys, but only three have appeared in humans this century. While some scientists have theorized that only these three can replicate in humans, this is probably an oversimplification. While phylogenetic analysis of the nucleotide sequence of the more conserved genes implies a definite host range for influenza viruses, the lineages are not that strict.

In aquatic birds, the viruses replicate primarily in the intestinal tract, less in the respiratory tract. Virus replicated in intestinal lining cells is fecally transferred into lakes by birds such as ducks. Birds that migrate north and south are more significant for influenza transmission than birds that migrate around the world.

This century has seen the Spanish, the Asian, the Hong Kong, and a Russian strain of influenza. The Spanish flu probably came from American soldiers in Europe or Chinese trench-diggers during the first World War. The Asian strain was a reassortment with three gene segments from aquatic birds and five segments from a previous human strain. Since it has been about 30 years since the last influenza pandemic, the influenza scientific community is expecting another one to emerge any time.

The hemagglutinin of human strains attaches to α2-6 galsialic acid. However, avian strains prefer α2-3 sialic acid. The pig has receptors for both α2-3 and α2-6. Furthermore, influenza strains from aquatic reservoirs (including hemagglutinin subtypes H2, H3, H4, H5, and H6) can replicate in pigs.

An influenza strain from 1979 was transmitted to pigs, reassorted with a human virus in the pig population, and became established in pigs. The fact that it became established is unusual. This virus did not become pandemic, because the human population already had antibodies to the H3 portion that was in the reassorted virus.

The molecular basis for the lethality of the 1918 virus is unresolved. Phylogenetic studies suggest that the virus came from an avian reservoir to humans with all its gene segments; so, it probably

was not a reassortment. Sequence analysis of a 1918 strain isolated from a paraffin block sample from a dead soldier confirmed that the virus came from pigs. However, it did not contain a series of basic amino acids that is believed to enhance pathogenicity. The sequence identified from the 1918 strain could have been that of a precursor virus that has since mutated.

Once in the human population, influenza viruses continue varying to give yearly epidemics. Each year, the CDC and the World Health Organization review influenza data from 120 labs around the world to design an annual vaccine. For the past several years, they have designed relatively successful vaccines. However, since 1972, 10 epidemics have caused more than 20,000 deaths a year in the United States. Moreover, during three years influenza epidemics caused more than 40,000 deaths annually in the U.S.

A normal non-immunocompromised person clears influenza virus in, at most, 7 days. In children receiving bone marrow transplants on immunosuppressive medications, virus can be shed for weeks or months. Hence, an enormous extension of viral replication, increased time for genetic changes, and increased morbidity and mortality may occur in patients receiving immunosuppressives for solid organ transplants.

Regarding xenotransplantation, pig organs used after initial early or acute infections would be a problem. Vaccination of the donor pigs, the human recipients, or the human staff is options. Vaccinating the staff could be particularly important: the deaths of some children receiving bone marrow transplants were traced to influenza viruses circulating in unvaccinated staff members. Multiple antiviral agents should also be made ready to combat infections in recipients.

Influenza specialists already anticipate another human pandemic. The question to be asked is whether xenotransplants from pigs, the natural influenza mixing vessel, would “facilitate the generation of the next pandemic strain.”

Spumavirus Infection Within and Across Species Lines

Drs. Arifa Khan and Walid Heneine spoke on this topic.

Dr. Khan spoke on ***Analysis of Foamy Virus Infection of Rhesus and Pig-tailed Macaques: Identification of Simian Retrovirus-Free Animals***. Foamy viruses belong to the group of spumaviruses in the classification of retrovirus. They are identified in electron microscopic (EM) analysis by their characteristically prominent envelope spikes. A high intracellular accumulation of particles is also associated with most infections. Eleven simian foamy virus (SFV) serotypes have been identified. Serotypes 1, 2, and 3 are prevalent in rhesus macaques, African green monkeys, and baboons. Baboons also have serotype 10, and chimpanzees have serotypes 6 and 7.

There is no evidence for naturally occurring human foamy viruses. All infections reported in people have resulted from cross-species transmission from nonhuman primates. The foamy virus that has been designated to be of human origin (HFV) probably actually came from a chimpanzee. When cross-species transmission does result in human infection, the retroviral sequences integrate stably into the human genome and can persist long-term.

SFV2 replication was compared in *Mus dunni* mouse fibroblast cells, Cf2Th dog thymus cells, Vero African green monkey cells, and HeLa human epithelial cells. The cytopathic effect (CPE) was slow in the primate cells, whereas the dog cells were highly susceptible to CPE. The development of CPE correlated with increase in RT activity. The mouse cells had the highest RT levels with all other cells studied having lower RT activity. Evaluation of SFV2 in human lung carcinoma A549 cells using an 8X-virus dose yielded an early CPE, but the RT level was still low. SFV2 infection of primary chicken embryo fibroblasts was also rapid, by CPE, but had low RT levels. Thus, SFV2 replicated most efficiently in *Mus dunni* cells.

Viral particles showed different accumulation patterns in *Mus dunni*, dog thymus and Vero cells. Infected *Mus dunni* cells had abundant intracellular and extracellular viral particles. Infected dog cells had high intracellular accumulation but a low number of extracellular particles. Infected Vero cells had few extracellular particles. EM analysis showed SFV2-associated apoptosis in HeLa and Cf2Th cells.

PCR primers and immunofluorescence assay (IFA) were developed to detect SFV infection in pig-tailed and rhesus macaques. Sequence analysis of multiple isolates revealed characteristic rhesus and pig-tailed viruses that differed from the SFV1 and SFV2 sequences. Interestingly, one group of negative animals was co-housed with positive animals for some period of time, but the group remained negative.

Studies are planned to evaluate the potential risk of foamy virus infections in humans. It appears that the virus can exist in an active and a latent state. It is recommended that animal handlers are regularly monitored for foamy virus exposure and simian-derived biological xenotransplant products are screened for the presence of foamy viruses.

Dr. Heneine spoke on ***Cross-Species Transmission of Simian Foamy Retroviruses to Humans Occupationally Exposed to Nonhuman Primates***. Two major groups of retroviruses infect humans. They are human T cell lymphotropic viruses (HTLVs) and human immunodeficiency viruses (HIVs). While the HTLVs were introduced into the human population thousands of years ago, the HIVs most likely entered the human population within the last century. There is no evidence that simian foamy viruses (SFV) have established in the human population. However, the use of baboons as xenotransplant donors would greatly increase human exposure to these viruses. Captive baboons are almost universally infected with SFVs. Yet, it is unclear what impact foamy virus infection has on humans or whether they can be transmitted between humans.

CDC has established a voluntary linked study with primate centers to identify transmission of simian retroviruses to primate handlers. The centers are surveying for SIV, STLV, SFVs, and Type D retroviruses. Foamy viruses are the most prevalent retroviruses among captive nonhuman primates, but they have no associated disease to date.

Data from 13 institutions in the United States and Canada yielded a seropositive prevalence among humans handling primates of 0.05 percent for SIV, 0.00 percent for STLV, and 1.8 percent for SFV. The four positive SFV individuals reported needle sticks or bites that may have been the source of exposure. The infecting SFVs probably originated in African green monkeys (one person) and baboons (three people). PCR detection of viral pol sequences was possible in the infected individuals PBLs. Yet, the spouses of three seropositive individuals remain seronegative

despite exposure for up to 19 years in one case, indicating that SFV is probably not sexually transmitted. However, it may take much longer to see evidence of sexually transmitted HTLV-1. At least, it would seem that transmission is not occurring readily through such routes as saliva.

Infection with STLV or SIV appears to be rare or absent in occupationally exposed humans; however, the roughly 2 percent prevalence of SFV in the same population may comprise the minimal level of infection anticipated among humans exposed to SFV infected xenografts. While SFV transmission to humans occurs without an abrupt change in pathogenicity, human susceptibility to these SFVs is now confirmed.

Panel Discussion III:

An open panel discussion with comments and questions from attendees ensued and the following points emerged. (1) The tropism switch for influenza from intestinal to respiratory occurs in the pig. (2) A study of 8,500 individuals, some in African populations in direct contact with pet monkeys has resulted in no evidence of widespread human seropositivity for foamy viruses. (3) Screening for other viruses, such as baboon endogenous retrovirus, among primate handlers is restricted by institutional and participant interest to partake in these tests. (4) The consequences of long-term viral shedding in immunosuppressed individuals should be studied in animal models. (5) Plans are under way to measure levels of SFV in body fluids such as semen and saliva. (6) It will be determined whether the higher number of humans infected with SFVs from baboons are indicative of greater susceptibility to baboon *versus* African green monkey SFVs, or whether this merely reflects more exposure. (7) Viral competition and replacement of one subtype over another and new viral host acquisition were discussed in the context of viral niches.

Factors Affecting Infectious Agent Pathogenicity

Drs. Jay Fishman, Francis Black, Julia Hilliard, and Jonathan Allan spoke on this topic.

Dr. Fishman spoke on ***Cytomegalovirus and Porcine Endogenous Retrovirus from Miniature Swine***. Host factors are likely to play a significant role in the expression and maintenance of infection by cytomegalovirus (CMV) and porcine endogenous retrovirus. The "net state of immunosuppression" is a major determining factor in assessing a recipient's infection risk following organ transplant. The dose, duration, and temporal sequence of immunosuppressive therapies primarily govern the immunosuppressive state. In addition, leukopenia, mucosal and skin surface barrier changes each influence a patient's susceptibility to infections. Also, recipient characteristics including immune defects, protein malnutrition, uremia, diabetes, and immunomodulating viral infections are certainly of consequence in overall risk of infection.

Infections occurring in the first post-transplant month are usually related to the surgical procedure itself. Subsequent complications include acute rejection and chronic rejection including graft injury. A timeline has been created that charts pathogens likely to emerge at various postoperative stages related to changes in immune deficiencies over time. If an infection becomes apparent that is inconsistent with the usual pattern of opportunistic infection, then it may reflect an unusual epidemiological exposure. These infections can be bacterial, fungal, parasitic, and viral.

CMV and Epstein Barr virus (EBV) are the major viral pathogens in allotransplantation. These viral infections cause problems in the recipient that are not just due to the infectious nature of the virus. They can lead to a viral "mononucleosis-like" syndrome in the transplant tissue, predispose the recipient to other infections, cause graft rejection, and/or lead to oncogenesis over a longer period of time (post-transplant lymphoproliferative disease; PTLD).

CMV can be activated from latency to an active infection by inflammation and cytokine release caused by other infectious agents, graft rejection, or immunosuppressive therapies. Also, activated CMV is capable of damaging the transplant tissue, making it more vulnerable to opportunistic infections and graft rejection. Porcine CMV can be reactivated in swine by immune suppression and isolated from the lungs (e.g., following bone marrow transplantation) of swine by performing a bronchoscopy.

Porcine CMV is characterized by intranuclear and intracytoplasmic inclusions, which are slightly larger than the inclusion typically seen in human CMV infections. Experimental attempts to infect human cell lines with porcine CMV and porcine cells with human CMV, however, have been unsuccessful, implying that interspecies protection against CMV infection exists. Thus, xenotransplantation may have an advantage over allotransplantation with respect to viruses that are rigidly species-specific.

Porcine endogenous retrovirus is constitutively expressed in normal miniature swine cells, particularly leukocytes. The virus is present in both activated and unactivated PBLs. Hence, it seems quite likely that it will be expressed in any transplanted organ. A variety of transcripts are found in different organs, probably due to alternate splicing. Full-length endogenous viral

sequences have been obtained from cDNA libraries from peripheral blood leukocytes of normal miniature swine (PERV-MSL). These had the greatest overall homology with gibbon ape leukemia virus (GALV), but they are typical Type C retroviruses. Multiple forms of PERV exist in single hosts.

In summary, a number of factors may increase or maintain viral transmission. Constitutive viral expression in the xenograft is very likely. Decreased MHC-linked immunity in the xenograft environment is also very likely and may contribute to the spread of viral infection once activated. The type and duration of immune suppression are critical. Any viral co-infections will be important. Virologists should further assess the effect of viral passage in host cells. And host and donor manipulations, particularly regarding the level of immune suppression, are likely to influence viral expression.

Dr. Black spoke on ***Hazards of Morbillivirus and Oncoretroviruses and Xenotransplantation***. While morbillivirus are generally host specific, there are latent examples of these viruses, and they have the ability to acquire increased pathogenicity when they are transferred from one host to another. Human immunity to measles, a morbillivirus, confers some immunity to other well-studied viruses in this group. However, if the measles vaccine is eradicated by the year 2010 as planned, then humans may become more susceptible to these pathogens.

Latency of morbillivirus is not usual, but may be a problem. Subacute sclerosing panencephalitis (SSPE) virus is a latent form of measles virus and represents the best-studied instance of morbillivirus latency in humans. Latency in this disease is thought to be due to an M protein mutation that makes virus replication inefficient and thus fail to elicit an adequate immune response in the poorly immune-surveyed brain tissue. The modified virus continues to replicate slowly, leading to death in about 10 years. Canine distemper, another disease caused by a morbilli virus, can remain latent in dogs for much of the animal's life, and this virus has caused very high mortality in other species as when it crossed into the Lake Baikal seals in the 1980s and into Serengeti lions in 1990s. The Australian "equine morbillivirus" that led to the death of 35 horses may actually be a Bat adapted virus that is latent in its natural host. Recognized latency of morbillivirus is thus of limited scope; but the persistence in this country, despite the high level of "herd" immunity, of small outbreaks of measles that cannot be related to importations, raises the possibility that latency of measles virus may be more common than realized.

It is not yet clear whether the only potential pig morbillivirus isolated from a peccary poses any threat to humans as recipients of pig organs. However, it is recommended that donor animals be tested for these viruses.

Oncoretroviruses represent another potential group of infectious agents with a long incubation period. Screening techniques for these viruses, of which HTLV-1 and HTLV-2 are the human varieties, are so species-specific that tests of donor tissue may not uncover more exotic varieties. HTLV-1 causes an adult T cell leukemia with a latency period of several decades.

Fortunately, Oncoretroviruses are not characterized by increased pathogenicity associated with host changes. Judging by interrelatedness of virus from diverse primates, interspecies transmission is not uncommon. It is easy to see how the virus might pass from monkeys to humans in the process of butchering the former for food. It is less obvious that pet monkeys may be infected by

humans when they are raised as pets. When these monkeys mature and return to the wild, they can transmit virus into the monkey population. The concern over humans getting virus from monkeys seems somewhat anthropomorphic in this speaker's view.

It is recommended that if primates are used as organ or tissue donors, they should not be taken directly from the wild. Instead, a controlled colony should be monitored over several generations to produce virus-free donors.

Dr. Hilliard spoke on ***Central Nervous System Viruses of Nonhuman Primates: Considerations of Donor Contaminants Transferred During Xenotransplantation***. Three viruses capable of causing central nervous system disturbances in native or foreign hosts were discussed. Assay development is under way for simian agent 8 (SA8), baboon cytomegalovirus, and reoviruses. While genetic variability is inherent in RNA viruses, genetic conservation prevails in DNA viruses. Regardless of the agent, extensive screening for these potentially fatal viral pathogens is an obligation of the scientific community, so that viruses can be identified if they attempt to jump hosts into the human population through transplant recipients.

While SA8 is a neurotropic alpha herpesvirus that originated with either baboons or African green monkeys, it is nearly always present in baboons. While it is fairly innocuous in its native host, SA8 has the potential to behave much like herpes B virus in foreign hosts, particularly immunocompromised or young animals. Herpes B is a macaque virus that is fatal in 80 to 90 percent of human cases. SA8 and herpes B are so conserved that their behavior and host response is essentially identical. SA8 antibodies are detectable by ELISA, and PCR analysis can be used to identify SA8. To give added assurance that a donor animal is SA8 negative, it will be important to rid any donor organs of all the baboon leukocytes acting as viral repositories. Nevertheless, long-term monitoring for latent virus will be essential for identifying any inadvertent zoonotic infections.

Baboon CMV is a beta herpesvirus. A recently isolated baboon-specific CMV isolates revealed a unique DNA insertion and outer envelope construction that was typical of human CMV isolates. Baboon CMV isolates exhibit readily apparent intra-strain variation. SA8 and CMV isolates grow in a variety of different culture systems, so that one cannot assume a sample is negative for virus with no growth in only a few cell types; multiple lines should be tested when attempting to isolate virus. Testing donor animals for antibodies by ELISA is also essential to complete the donor profile. So far, long-term animal workers do not have antibodies to SA8 or baboon CMV. However, ELISA is not always conclusive and more sensitive PCR detection should be included in the future.

A novel reovirus was recently identified when nine potential donor baboons developed encephalitis in a capture colony of baboons used for medical research. The isolated reovirus appears unique to baboons, but it shows the great similarity to rattlesnake reovirus. Baboons appear to be globally exposed to this virus and generally carry it without effect, but small pockets of “an unusual clinical syndrome” emerge.

Baboons have been organ donors since 1964 and it is possible that they could be used again in highly selective cases. They could be used when a bridge transplant is needed for a pediatric patient or someone waiting for a human organ. To this end, “Specialized specific pathogen-free

animals are currently being monitored at different locations for potential donor service." Yet, a number of biosecurity and maintenance challenges remain even after the animals have been screened.

It remains unclear how long xenograft recipients should be screened for potential viral infections. Consensus does not exist whether they should be screened for 6 months or less or 10 years or more. While herpes B is usually fatal in human, some patients survive with early antiviral therapy and still others appear to have withstood infection without intervention. The behavior of this one agent should give the biomedical community a dramatic reason to ponder the possibilities of xenotropic viral infection, even when remote. One goal that should be met is to understand viral pathogenesis in the native host and to inform physicians of what symptoms to look for. The post-transplant physicians will be the vanguard serving to identify post-transplantation virus infectious threat to the individual recipient, close contacts, hospital workers and the population at large.

Dr. Allan spoke on ***Simian Retroviruses of Baboons: Implications for Xenotransplantation***. Baboons and pigs have been proposed as donor species for xenotransplantation, yet the public health risks associated with these species are quite different. There is a greater likelihood for transmission and establishment of simian viruses, because the close genetic relationships between baboons and humans result in a high degree of conservation in viral receptors, thus allowing for infection. Nonhuman primates are not domesticated and as such carry viruses that may be only once removed from wild populations. Furthermore, colony-bred animals continue to circulate resident viruses within their captive populations. In addition, new emerging infections are being discovered in baboons as recently evidenced by the discovery of a neuropathogenic reovirus in baboons. Of importance is the notion that retroviruses and herpesviruses have "long fuses," and thus transmission may not become apparent for several years. Examples included HIV-1 and the AIDS epidemic, which is generally considered to have arisen by cross-species transmission of SIVs to humans. Most commonly used methods to limit the spread of infectious diseases including barrier containment and quarantine for both the donor species and recipient are unlikely to be effective for retroviruses, since the use of quarantine is only useful for infectious disease where clinical signs are seen within the quarantine period.

Scientists cannot fully predict whether a particular virus, once transmitted to humans, will be pathogenic and predictions cannot be made with certainty based on how the virus affects its natural host. Many naturally occurring viruses commonly fail to induce disease in their natural nonhuman host but can result in severe disease in humans; historical evidence has shown this with AIDS, herpes B virus infection from macaques, and leukemias caused by human T cell lymphotropic virus from African primates. The fact that a virus causes no disease in the donor species has little bearing on whether it can become a public health problem in the human recipient. Beyond the recipient, the greatest concerns relate to dissemination of those microbes into the general population. For both known and unknown viral infections that are persistent infections, the possibility of transmission increases with the number of transplants performed. Technologies and surveillance methods are not well adapted to prevent transmission and possible contamination of the blood supply. Just as HIV-1 was spread through blood transfusions, simian retroviruses are poised to be transmitted in similar manners, either sexually or via blood transfusions. In cases where the infection is silent and not yet characterized, identification of such an infection generally

requires evidence of disease. In the worst case scenario, this could happen several decades after the transplants.

Baboons harbor several known viral infections that are potentially dangerous in the transplant setting. At least three herpesviruses (SA8, CMV, and herpes papio) are endemic in baboons, and both endogenous (BaEV and SERV) and exogenous retroviruses (STLV, SFV, Type D) are commonly found in baboons. For example, baboons carry STLV, with upward of 80 percent of them persistently infected in U.S. colonies. In addition, evidence of lymphoid cancers associated with infections is seen in 1 to 4 percent of infected animals, but only after decades. It has been suggested that the human version HTLV-1 arose by cross-species transmission from African primates including baboons. Since retroviruses can remain latent in the host by nature of their replication cycle, the introduction of a known or unknown retrovirus into humans' means that infection, if transmitted, will become endemic in humans. The endemic nature of infection will depend on several factors, including route of infection and the relative levels of viremia.

A second simian retrovirus that must be considered in the transplant setting is SFV, another virus with high rates of infection in adult baboons (99 percent). While no disease association is apparent, it has been shown to infect humans and the long-term consequences of transmission to humans are unknown. In baboons, the viruses have a wide range of cell tropism and are highly cytopathic in human cell lines. In a retrospective study of tissues from two human recipients of baboon livers, evidence of SFV, an endogenous retrovirus (BaEV), and even baboon mitochondrial DNA was observed in a range of tissues including liver, lymph nodes, and kidneys, suggesting the persistence of baboon cells circulating in the human recipient throughout the post-transplant period. Called microchimerism, the persistence of lymphocytes during the life of the transplant recipient suggests that persistence of any viral infections carried by those cells is highly likely.

Finally, draft guidelines meant to deal with possible infectious disease risks have focused primarily on containment; those interventions after viruses have been transmitted to human recipients. The detection of either new viral infections or ones that are known to exist in the donor species in the transplant recipient will be difficult to address if disease manifestations are delayed for decades. Furthermore, assay development for simian viruses has been slow. The first priority must be to protect public health, and nonhuman primates are relatively risky in light of the range of persistent viral infections. While transplant donor resources are scarce, providing even a few "clean" nonhuman primates is very expensive. Therefore, greater attention should focus on the porcine resource, which has a very large potential benefit and relatively low infectious disease risk in comparison to the baboon donor.

Panel Discussion IV:

An open panel discussion with comments and questions from attendees ensued. (1) CMV-related infection occurs in roughly 50 percent of allotransplant recipients. It would be preferable to remove CMV from xenotransplant donor herds as well, because it is likely to cause graft damage. (2) Baboon CMV has not yet been tested for susceptibility to the antiviral ganciclovir. (3) It is unclear what advantages encapsulated cells like neural cells and pancreatic islet cells may have, unless they are shown to be protected from the allogeneic or xenogeneic response. Viral load is likely to be an important determinant for the level of immune suppression. (4) Some herpes B

virus infection survivors are back out in the community. Some are merely antibody positive and have never had a documented clinical episode. HLA typing has not been done on these individuals. Recommendations for ways that these individuals should limit themselves were published in “Guideline on Evaluation of People Potentially Exposed to or Infected With B Virus” in the February 1995 issue of *Clinical Infectious Diseases*. Survivors usually demand confidentiality and do not want to be stigmatized as “carriers.” (5) The extensive diversity among simian and human retroviruses does provide some evidence of human to nonhuman primate transmission.

Plenary Discussion Future Directions

Drs. Stanley Weiss, Daniel Salomon, Ron Ferguson, John Coffin, Jay Fishman, and Jeffrey Platt comprised a panel focusing on how the information provided about cross-species infectivity and pathogenesis at this meeting will affect the course of xenotransplantation.

Dr. Weiss, the panel moderator, began by pointing out that the goal of this discussion was not to formulate policy, but to provide "specific guidance regarding the scientific issues and problems that remain" for xenotransplantation. Scientific advice is needed on how to approach the critical issues that must be processed before xenotransplantation continues.

Dr. Ferguson, president of the American Society of Transplant Surgeons, emphasized that this meeting has allowed for dialogue to develop between clinicians and scientists, who have traditionally approached the issue of xenotransplantation from "two quite different worlds." A goal is to facilitate a responsible and safe approach to xenotransplantation. Clinical trials will be established at researchers' comfort level of the unknown, in an attempt to reconcile "fear of the unknown" with "the practical reality of what could happen." The comfort level of clinical trials will be greater if contingency plans, such as making vaccines available and standards for serological and molecular testing are in place. Furthermore, dialogue between and among industrial, scientific, clinical, and regulatory communities will facilitate xenotransplantation implementation.

Dr. Salomon, a transplant physician, identified the field of transplant medicine as one in which new fields continue to intersect. Retrovirology, infectious disease biology, veterinary medicine, and microbial ecology all coincide here. Transplant physicians and surgeons will have to "roll with the punches" introduced from these disciplines as they merge with transplant medicine. Discussions on how to do xenotransplants responsibly have been ongoing for at least 3 years, and the time is imminent when clinical trials with pig organ transplants into humans could begin.

In preparation for xenotransplant trials, more studies on primary human cells, not just cell lines, need to be performed to test for their ability to be infected. The cell tropisms for zoonotic infections need to be clarified. Specific risk assessments for viral recombination events need to be done. It is important to get more exact information on how likely it is that endogenous retroviruses will actively replicate after transplantation; contrasting opinions have emerged on this issue. Moreover, if patients are infected by endogenous retroviruses, will this cause disease? If patients become viremic, what antivirals are available for them? Do the risk profiles for infectious disease of pigs or primates indicate that only one or both are acceptable as donor animals? Finally, when clinical trials begin, it will be critical to have in place mechanisms to closely monitor patients and primary contacts with the expert support and oversight to promptly detect and alter or stop trials if any unusual risk or complication is identified.

Dr. Coffin, a virologist, affirmed that virological knowledge should play a key role in the shaping of xenotransplantation "to bring this technology to complete utility." Pathogenic risks of many viral infections can be quantified, but they may not be immediate; they may materialize years after transplantation. However, different endogenous viruses do different things. Some viruses can

cause viremia that goes unnoticed in chickens. Some mice contract certain viral infections that cause predictable death in a laboratory setting, but the same viral infection may run a different course in nature. Endogenous viruses may be less harmful than a newly emerging exogenous virus that crosses host species barriers. Yet, all in all, it is still difficult to define precisely the infection risk due to a particular xenotransplant until it is actually done.

The most serious, albeit less likely, event is the potential to generate a new epidemic by introducing nonhuman infectious agents into the human population. A number of the harmful viral infections that are documented in science and in the press come from "relatively benign host-virus relationships in various species of animals." Such acquisition of human hosts by a virus requires an environment conducive for infection, transmission, and establishment of the zoonotic infection in the host. The ability of the new virus to be transmitted between humans is a major concern. Fortunately, regulations can be set up to prevent xenotransplant recipients from transmitting virus through means such as donating blood. It would be very helpful to use an animal model to test xenotransplantation.

Dr. Platt, a transplant immunologist, referred to immunology as the last line of defense against infection and pointed out that immunologists, virologists, and infectious disease specialists are in an analogous position to contribute to the final plans to proceed with xenotransplantation. Immunology has always been the final hurdle in transplant technology. Although xenotransplantation will not be a widespread practice for some time, the scientific community shares some of the responsibility for shaping how medical science proceeds with it. Preclinical experiments must help establish what major remaining immunological barriers must be overcome for this technology to proceed successfully. Moreover, hundreds of millions of people around the world who are in close contact with pigs every day and have been exposed to pig blood through scratches or other means can provide much-needed information on cross-species infectivity from pigs.

Xenotransplanted organs may actually confer advantages to the host defense of their recipients. Cytokines responding to xenostimulation may actually augment the battle against an infection to a degree not provided by a lower level response initiated in response to allostimulation. Xenostimulation may bring out "bigger guns" to suppress infection. In addition, since zoonoses are likely to occur regardless of whether xenotransplantation proceeds, xenografts could theoretically provide a tool to combat zoonotic infections or environmental catastrophes of various types. Indeed, the several xenografts performed in the early 1990s were motivated in part by resistance of animal cells to infection by human pathogens. Furthermore, future xenotransplants could be used to "piggy-back" other biotechnology techniques, including gene therapy, into recipients to combat infectious and immunological conditions.

Dr. Fishman, a transplant infectious disease expert, expressed the importance of putting the individual and community risks associated with xenotransplantation "in terms that the public can grapple with." To provide sound recommendations, research that measures these risks and advantages will need federal and corporate funding and public dissemination with education. Appropriate animals models (e.g., immunosuppressed xenograft recipients) are critical to determine the risks associated with specific organ transplants from specific animals. Cooperation between corporate entities and multiple laboratories will accelerate technological development in

this field and create the kind of open research environment that is more likely than an environment wrought with competitive secrecy to convince the public that the science is sound.

Panel Discussion V:

An open panel discussion with comments and questions from attendees ensued. (1) There is no longer a question of whether xenotransplantation will proceed; instead the question is how fast and what procedures will government it's safe conduct. The issue at hand is to make it as safe as possible. Additionally, primates should not be ruled out altogether, since they have been the most successful xenodonors for humans to date. However, caution must be exercised when using organs or tissues or cells from non-human primates (or for that matter any animal donor) and all possible measures must be taken to ensure that the possibility of transmission of infectious agents be as close to zero as possible. (2) Simple recombinant protein vaccines are a potential contingency tool that could be tried in an initial clinical trial if recipients developed a viral infection. Vaccines for porcine endogenous retrovirus could be used in this way. (3) Vaccinating donor animals against their own endogenous viruses is unnecessary, since they are not susceptible to them. (4) Even though neutralizing antibodies, which are now called natural antibodies, have been ineffective against some viral agents such as HIV, it would still be useful to do the serological experiments to determine whether these antibodies can naturally neutralize certain viral agents. (5) Porcine reproductive respiratory syndrome (PRRS) virus appeared some time between 1980 and 1985. It is a single-stranded RNA virus, replicates in macrophages, causes spontaneous abortions in pregnant pigs, and appeared in Europe and the United States about the same time. A third form has emerged in the past 6 months. (6) Consensus does not exist over whether renal failure patients will even respond immunologically to vaccines, since studies with a hepatitis vaccine had limited success in this population. (7) Time constraints surrounding many transplant surgeries emphasize the need to develop more rapid diagnostic assays for infectious agents. (8) The success rate in allotransplants has motivated xenotransplant enthusiasm; as few as 15 to 18 percent of recipients experience an acute rejection episode. (9) Invasive testing, such as spinal taps, may be warranted to test donor animals for infectious viral agents. (10) Cellular tropism may not be easy to assess fully, because one cannot test all the many cell types with various receptors throughout the body. (11) Currently, hepatitis C virus is knowingly transmitted to kidney transplant recipients who may then get liver disease in 20 years instead of dying in 2 years from their original disease. (12) An “international collaborative interdisciplinary registry” of all xenotransplant recipients was proposed to track potential outcomes over the long term. (13) “Go/no go” assessment points could be written into an initial prospective trial such that if the first group of transplant recipients were, hypothetically, all viremic at 12 months post-xenotransplantation, then the xenotransplants would stop until a way around the problem was devised.

Dr. William Raub gave the closing remarks. The cooperation among four components of the U.S. Public Health Service to sponsor this meeting was impressive. The topics covered will help refine draft guidelines for xenotransplantation. Clear communication to the public about what is going on is crucial, since the taxpayers support much of the basic research that will help xenotransplantation proceed responsibly. The speakers provided a “framework for risk assessment at a protocol-specific level” to guide scientists, clinicians, and institutional review boards. Rational risk assessment is the best precursor to further development in this field. A follow-up workshop

was envisioned to pinpoint exact guidelines for xenotransplantation. Dr. Raub thanked the participants for their productive input.

Whereupon, the meeting was adjourned.